Essential stations in the intracellular pathway of cytotoxic bovine seminal ribonuclease[1]

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Bovine seminal RNase (BS-RNase) is a dimeric RNase selectively cytotoxic for malignant cells. No information is available on its pathway from the extracellular matrix through the cytosol, where it degrades rRNA. An investigation of this pathway is reported here, carried out by immunofluorescence studies, by assessing the effects on BS-RNase cytotoxicity of drugs that affect specific intracellular compartments and by assaying the behaviour of a protein variant, BS-RNase-KDEL (BS-RNase in which a Lys-Asp-Glu-Leu peptide segment is inserted at the C-terminal ends of the subunit chains), endowed with a consensus sequence that directs proteins to the endoplasmic reticulum. BS-RNase was found to bind both normal and malignant cells and to be internalized by both cell types in endosome vesicles. Non-cytotoxic RNases, such as RNase A and a monomeric derivative of BS-RNase, did not bind to the cell surface and were not internalized. However, an engineered, dimeric and cytotoxic variant of RNase A bound effectively and permeated cells. The results of immunofluorescence studies, the effects of nigericin, monensin and brefeldin A on the cytotoxic action of seminal RNase, and the behaviour of the BS-RNase-KDEL variant, led to the conclusion that the pathway of BS-RNase in malignant cells from the extracellular matrix to the cytosol has two essential intracellular stations: endosomes and the *trans*-Golgi network. In normal cells, however, the protein does not progress from the endosomal compartment to the Golgi complex.

Key words: anti-tumour, cytotoxins, endocytosis, intracellular traffic.

INTRODUCTION

Seminal RNase from bovine seminal vesicles (BS-RNase; see [1] for a review) is an unusually dimeric RNase from the pancreatic-type superfamily. The two identical subunits are held together by non-covalent forces and by two intersubunit disulphides. First proposed as an antitumour agent by Matousek [2], it has since been shown, by *in vivo* and *in vitro* studies (reviewed in [3]), to have a powerful cytotoxic action surprisingly selective toward malignant cells.

Monomeric derivatives of BS-RNase are active enzymes, but are not cytotoxic [4]. Monomeric RNase A from bovine pancreas, the superfamily prototype, with more than 80% of its amino acid sequence identical with that of BS-RNase subunit, is not cytotoxic. However, when engineered into a dimeric protein [5] or rendered partially insensitive to the cytosolic RNase inhibitor [6], RNase A becomes cytotoxic. Recently, human pancreatic RNase, also monomeric and devoid of any bioactions other than its catalytic action, has been engineered by different strategies into a cytotoxic RNase [7,8].

Structural determinants of the antitumour activity of BS-RNase, and essential steps in its mechanism of action, have been elucidated. It has been found that prerequisites for the cytotoxic action of the enzyme are its catalytic activity and dimeric structure [4], and the saturable reversible binding of the enzyme to specific sites on the extracellular matrix [9]. It has been reported that retinoic acid greatly increases the cytotoxic action of the protein by severely affecting the Golgi apparatus [10]. It has been also reported that BS-RNase blocks protein biosynthesis by degrading rRNA [9], an effect exerted by the enzyme only in malignant cells.

Other homologous cytotoxic RNases have been isolated from amphibians [3], one of these being Onconase™ from the leopard frog (*Rana pipiens*), which has been investigated in detail and also tested in clinical trials [11]. As for its mechanism of action, Onconase™ must be catalytically active [10,12,13] to exert its cytotoxic action. The protein enters the cell by binding to receptor-like sites on the cell surface and is routed through the Golgi apparatus to the cytosol, where it degrades tRNA [14], rather than rRNA, as reported for BS-RNase.

A much larger body of mechanistic information is available for plant and bacterial cytotoxins, such as diphtheria toxin, cholera toxin, and *Pseudomonas* exotoxin [15]. They all enter the cell through receptor-mediated endocytosis, reach the endosomal compartment, and all eventually reach the cytosol. Here they exert their diverse enzymic actions on different components of the protein-biosynthetic machinery, which results in cell death. However, they may reach the cytosol through different pathways, either through a direct translocation from endosomes, as in the case of diphtheria toxin, or through the Golgi apparatus and

Abbreviations used: BS-RNase, bovine seminal RNase; BS-RNase-KDEL, BS-RNase in which a Lys-Asp-Glu-Leu peptide segment is inserted at the C-terminal ends of the subunit chains; BFA, brefeldin A; ECM, extracellular matrix; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; DMEM, Dulbecco's modified Eagle's medium; MCM-BS-RNase, catalytically active monomeric derivative of BS-RNase; PLCC-AA-RNase, a dimeric variant of RNase A ([A19P,Q28L, K31C,S32C]RNase A); A19P, Ala[19] → Pro etc.; FRTL-5, a Fischer-rat thyroid cell line; TK-6, Fischer-rat thyroid tumour cells derived from a rat thyroid follicular carcinoma; MPTK-6, a cell line derived from Fischer rat lung metastases of the TK-6 tumour.

[1] This paper is dedicated to the memory of Mariarosaria Mastronicola, who died suddenly in September 2000.

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the endoplasmic reticulum (ER), as observed for other toxins, such as ricin.

Here we report the results of a study aimed at tracing the path of BS-RNase to the cell cytosol.

EXPERIMENTAL

Materials

BS-RNase was purified as previously described [16]; RNase A (type XII), brefeldin A (BFA), monensin and nigericin were purchased from Sigma. The catalytically active monomeric derivative of seminal ribonuclease (MCM-BS-RNase) was prepared by selective reduction of the intersubunit disulphides, followed by alkylation of the exposed thiol groups [17]. PLCC-AA-RNase, a dimeric variant of RNase A ([A19P,Q28L, K31C,S32C]RNase A), was prepared as described in [5]. Polyclonal antibodies against BS-RNase or RNase A were obtained from rabbits following standard procedures [18] and used at a dilution of 1:1000. The monoclonal antibody against TGN38 [19], a *trans*-Golgi-network (TGN) resident protein, was obtained from Affinity BioReagents via Vinci-Biochem, Florence, Italy. Fluorescein- and rhodamine-tagged goat anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch, West Grove, PA, U.S.A., and used at dilutions of 1:50 and 1:30 respectively. The Sequenase Sequencing Kit and labelled nucleotides were purchased from Amersham Pharmacia Biotech. Wizard DNA Purification Kit for elution of DNA fragments from agarose gels, enzymes and other reagents for DNA manipulations were from Promega Italia, Milan, Italy.

Cell cultures

Simian-virus-40-transformed mouse fibroblasts and the parental non-transformed Balb/C 3T3-line were obtained from the A.T.C.C. (American Type Culture Collection, Manassas, VA, U.S.A.) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco/Life Technology) supplemented with 10% (v/v) fetal-bovine serum (GIBCO/Life Technology). Fischer-rat thyroid cells (FRTL-5) were grown in continuous culture in Ham's F12/Coon's modified medium (GIBCO/Life Technology), supplemented with 5% (v/v) calf serum (GIBCO/Life Technology) and a mixture of growth factors as described in [20]. Fischer-rat thyroid tumour cells (TK-6), derived from a rat thyroid follicular carcinoma, and a cell line (MPTK-6) derived from Fischer rat lung metastases of the TK-6 tumour, were grown in the same medium as FRTL-5, but without the growth factors [21].

All media were supplemented with 4 mM glutamine, 400 units/ml penicillin and 0.1 mg/ml streptomycin. All cell lines were maintained at 37 °C in a humidified incubator containing 10% CO_2 mixed with air.

Protein-synthesis assay

A standard procedure [22] was followed, with modifications. Briefly, cells were plated in 24- or 96-well plates at the specified cell densities in DMEM complete medium. After treatment with the appropriate effector, the medium was removed and the cells were incubated in leucine-free RPMI-1640 medium (ICN) without fetal-bovine serum in the presence of 1 μCi of [^{3}H]leucine/well. After 1 h the medium was removed and the cells were precipitated with 5% (w/v) trichloroacetic acid, washed three times with ethanol, dried and solubilized in 0.1 M NaOH containing 0.1% SDS. The acid-precipitated radioactivity was measured in an LS 1701 liquid-scintillation counter (Beckman Analytical). Incorporation values (means for triplicate samples) were expressed as percentage of [^{3}H]leucine incorporation with respect to untreated cells.

Immunofluorescence studies

Mouse fibroblasts (3T3 and SVT2 cell lines) and rat thyroid cells (FRTL-5, TK-6 and MPTK-6 cell lines) were seeded on glass coverslips in 24-well plates and grown to semi-confluency. The cells were washed with 1% BSA in PBS, then incubated with the RNase under test in complete medium. After incubation, cells were washed with 0.1% BSA in PBS, and then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. Fixed cells were incubated with primary antibodies (anti-RNase serum) for 20 min at room temperature in a humidified chamber, then washed with 0.1% BSA in PBS and incubated with fluorescein- or rhodamine-conjugated secondary antibody under the conditions described for the primary antibodies. Finally, treated cells were washed again with 0.1% BSA in PBS, rinsed with water, then mounted in 50% glycerol in PBS. To investigate internalization of RNases after incubation as described above with the appropriate protein, cells were washed with 1 M Hepes, pH 7.5, containing 0.1 M NaCl (Hepes/NaCl) for 5 min, then fixed and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Treatment with primary and secondary antibodies in succession was as described above. Samples were examined by epifluorescence using an Axiophot microscope (Zeiss).

For experiments involving co-localization, 3T3 and SVT2 cells were fixed and permeabilized as described above, then treated in sequence, as described above, with (1) anti-BS-RNase serum, (2) anti-rabbit fluorescein-conjugated secondary antibody, (3), anti-TGN38, a monoclonal antibody, at a dilution of 1:100, (4) an anti-mouse rhodamine-conjugated secondary antibody. The treatment with the anti-TGN38 antibody was carried out for 18 h at 4 °C. The results of these experiments were examined by confocal microscopy. Cells were examined using an Olympus IMT-2 microscope equipped with an MRC-1024 laserscan confocal system (Bio-Rad) using the 488 nm and 568 nm excitation lines from an argon/krypton laser. Green and red emissions were collected and processed with the Lasersharp 1024 software (Bio-Rad).

General procedures for DNA manipulation

Bacterial cultures, plasmid purification and transformation were performed as described by Sambrook et al. [23]. Double-stranded DNA was sequenced using the dideoxy method of Sanger et al. [24] and the Sequenase Sequencing Kit.

Expression and purification of BS-RNase-KDEL

The cDNA encoding the BS-RNase subunit chain [25], cloned between the *Nde*I and *Bam*HI restriction sites in vector pET22b(+), was mutated at the C-terminus and amplified by PCR. The oligonucleotides, synthesized by Ceinge (Naples, Italy), were: 5′-CCGGAATTCCATATGAAAGAAAGCG-3′ and 5′-CGCGGATCCCTAGATCTACAGTTCGTCCTTCACTGA-AGCATCGAA-3′. The latter, overlapping with the cDNA at the 3′-OH, included a 12-base insertion corresponding to the four codons (underlined) encoding the peptide Lys-Asp-Glu-Leu (KDEL). The PCR was performed with a PerkinElmer–Cetus DNA thermal cycler under the following conditions: 30 cycles each including 2 min at 95 °C, 2 min at 50 °C, 2 min at 73 °C. After the last cycle, the sample was kept for 10 min at 73 °C, then stored at 4 °C. The amplified PCR product was identified by agarose-gel electrophoresis to be a DNA fragment of about 400 bp. It was extracted and purified from agarose gel, cloned

into pET22b(+) plasmid (Novagen via Merck Eurolab, Milan, Italy) between *Nde*I and *Bam*HI restriction sites, and sequenced. The final sequence was that designed to generate a BS-RNase variant (BS-RNase-KDEL) containing a tail of Lys-Asp-Glu-Leu at its C-terminal end.

The BS-RNase-KDEL cDNA was then amplified in *Escherichia coli* JM101 cells (Boehringer) and expressed in BL21-DE3 *E. coli* cells (AMS Biotechnology). For expression, cells were grown to an attentuance (D_{600}) of about 3.0 in Terrific Broth and induced with isopropyl 1-thio-β-D-galactopyranoside. After overnight incubation, cells were collected by centrifugation and the protein was purified from inclusion bodies as previously described for BS-RNase [25]. The resulting protein was treated with *Aeromonas proteolytica* aminopeptidase (Sigma) for removal of Met^{-1} (the initiator methionine residue). The BS-RNase-KDEL variant was found to be homogeneous by SDS/PAGE and with an RNase activity, as determined in parallel Kunitz assays [26], comparable (within 10%) with that of wild-type BS-RNase (46 ± 5 Kunitz units/mg of protein).

RESULTS AND DISCUSSION

Immunofluorescence studies

3T3 mouse fibroblasts, and the 3T3-SVT2 malignant cells derived from the 3T3 line by simian-virus-40 transformation, make up a convenient experimental system for the study of selective cytotoxicity of BS-RNase [9]. The experiment illustrated in Figure 1 is representative of a series of experiments performed with several preparations of enzyme. Exponentially growing cells were treated after 24 h of growth with the protein (50 μg/ml) for 75 min at 37 °C, then tested with anti-BS-RNase antiserum as described in the Experimental section. BS-RNase was found to bind the cell surface of both 3T3 and SVT2 cells (see Figures 1A and 1C). Identical results were obtained (results not shown) when treatment with the ligand was reduced to 5 min or extended to 24 h. The fluorescence could be readily and quantitatively removed by treating the cells with a Hepes/NaCl solution (1 M Hepes containing 0.1 M NaCl at pH 7.5).

To investigate the intracellular localization of BS-RNase, exponentially growing 3T3 or SVT2 fibroblasts were treated with BS-RNase as described above, then stripped of surface-bound proteins with the Hepes/NaCl solution, and treated with 0.1% Triton X-100 to render them permeable to the anti-BS-RNase antiserum. The results of a typical experiment, in which cells were treated with BS-RNase for 75 min, are shown in Figures 1(B) and 1(D). BS-RNase is found to be localized intracellularly in vesicles, both in perinuclear regions and throughout the cytoplasm. When the treatment with BS-RNase was prolonged (e.g. to 24 h), the protein was found to be localized to larger vesicles, but only in malignant SVT2 cells.

When 3T3 or SVT2 cells were treated with BS-RNase at 4 °C, binding at the cell surface was not affected, but no internalization of the protein was observed when these cells were permeated by anti-BS-RNase antibodies (results not shown). These results suggest that the process of internalization is temperature-dependent.

Similar results were obtained with an analogous set of normal and malignant cells of epithelial origin. The cells tested were from rat thyroid, namely a normal stabilized line (FRTL-5 cells)

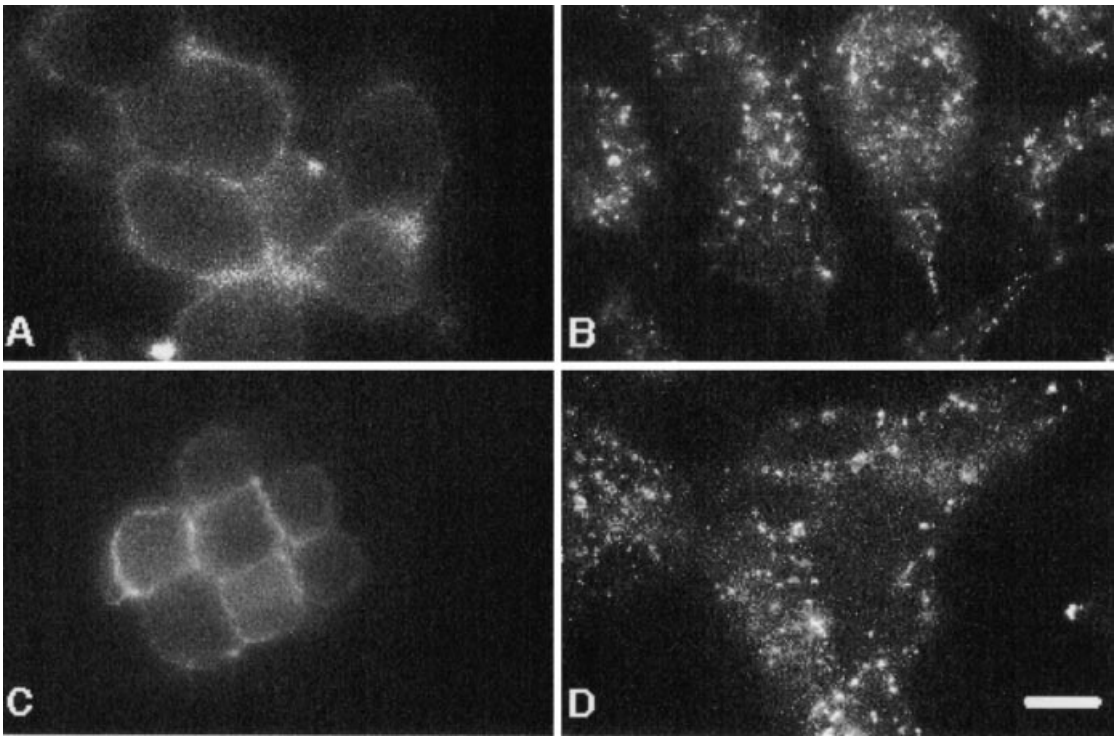

Figure 1 Fluorescence studies of cell binding and internalization of BS-RNase in mouse fibroblasts

(**A** and **B**) 3T3 fibroblasts; (**C** and **D**) transformed SVT2 fibroblasts. Cells were treated with BS-RNase (50 μg/ml) for 75 min at 37 °C, washed with BSA and then fixed in formaldehyde without permeabilization (**A** and **C**) or after a high-salt washing and permeabilization with Triton X-100 (**B** and **D**). Cells were then incubated with anti-BS-RNase serum for 20 min at room temperature, followed by incubation for 20 min with fluorescein-tagged goat anti-rabbit secondary antibody. The bar represents 10 μm.

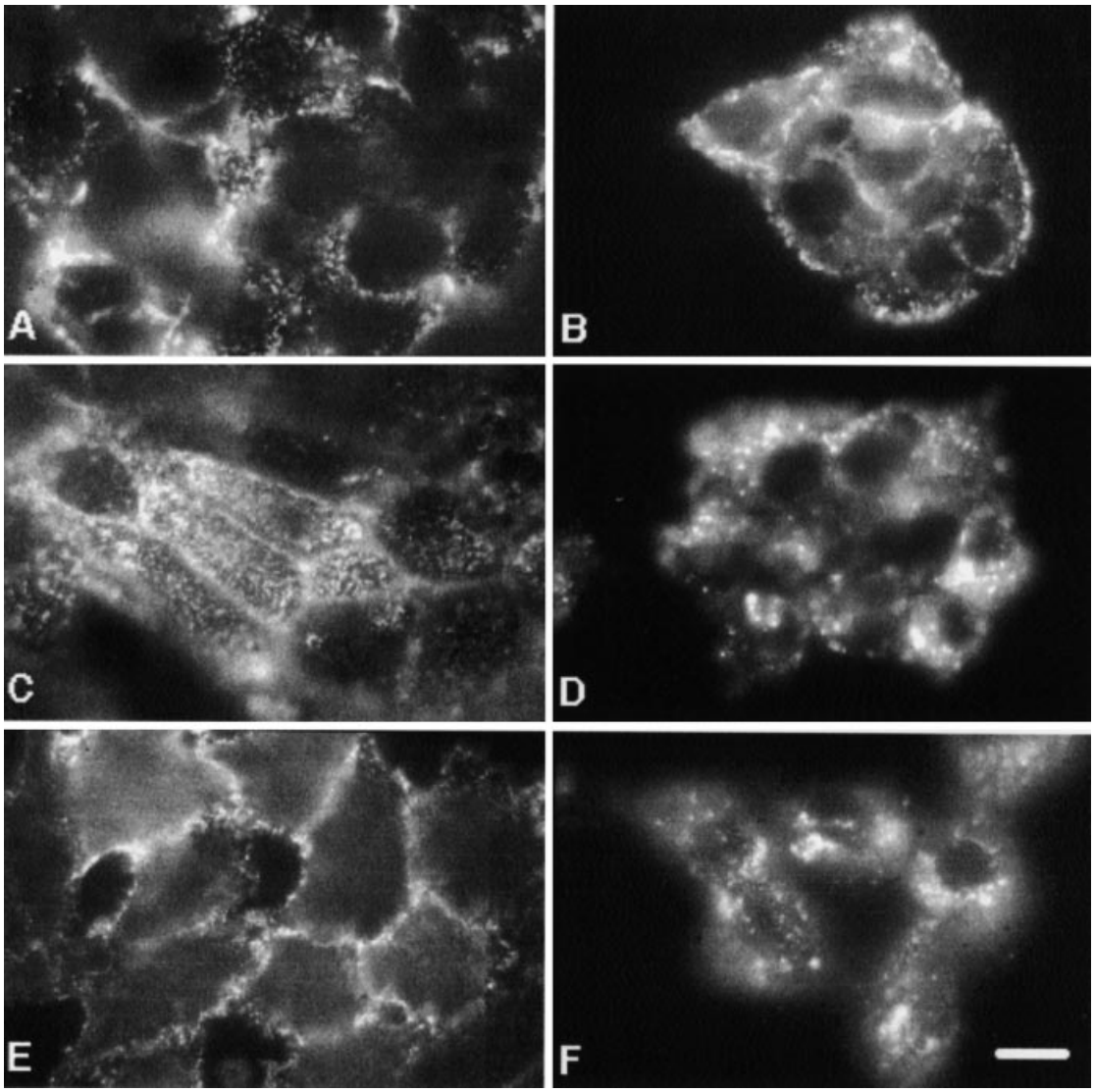

Figure 2 Fluorescence studies of cell binding and internalization of BS-RNase in rat thyroid cells

(**A** and **B**) FRTL-5 cells; (**C** and **D**) TK-6 cells; (**E** and **F**) MPTK-6 cells. Cells were treated as described in Figure 1: without permeabilization (**A**, **C** and **E**), or after high-salt washing and permeabilization (**B**, **D** and **F**). The primary antibody was anti-BS-RNase serum; a rhodamine-tagged goat anti-rabbit antibody was used as secondary antibody. The bar represents 10 μm.

and two malignant cell lines, one derived from a follicular carcinoma (TK-6 cells), the other (MPTK-6) from lung metastases of the TK-6 tumour. Identical results of a surface localization and internalization in vesicles were obtained, as shown in Figure 2. In this case, the fluorescent surface signals were clearly associated with extracellular matrix (ECM) components. Again, no differences were detected in fluorescence signals between normal and malignant cells. When the cells were permeabilized to allow internalization of anti-BS-RNase antibodies, in these cell types, too, the protein was found to localize in endosome-like vesicles (see Figures 2B, 2D and 2F).

Negative controls for the immunofluorescence experiments were carried out under identical conditions using RNase A, a homologous RNase that does not react with the anti-BS-RNase antibodies, and a monomeric derivative of BS-RNase, which is instead as immunoreactive to anti-BS-RNase antibody as the native dimeric enzyme. In either case, no fluorescent signals were detected (results not shown).

The results of previous studies [9], carried out with 3T3 and SVT2 fibroblasts, as well as with Chinese-hamster ovary cells, have shown that specific, saturable and reversible binding sites for BS-RNase are located in the ECM. These data and the results reported above lead us to conclude that the first station of BS-RNase in its journey towards the cell cytosol of malignant cells is at the ECM. The detection of internalized protein in vesicles, and the lack of cellular uptake of the protein at a low temperature, lead us to conclude that the second station is endosomal. This, however, may not exclude that the fluorescence signal extends also to vesicles that belong to the Golgi structure (see below), especially after a prolonged treatment with the enzyme, as reported above.

It is surprising to find that the protein follows an identical ECM–endosome pathway in both normal and malignant cells, whereas the protein reaches the cytosol only in malignant cells, where it exerts its catalytic degradation of rRNA [9]. The mechanism by which the protein is internalized is also mysterious.

Table 1 Effects of monensin and nigericin on the toxic effect of BS-RNase

SVT2 cells were plated in DMEM complete medium at 5×10^3 cells/well in 96-well plates. After 24 h of growth, cells were treated with BS-RNase (50 μg/ml) for 16 h at 37 °C in the absence or the presence of 100 nM monensin or 10 nM nigericin. After treatments, the medium was removed and cells were incubated in serum- and leucine-free RPMI-1640 medium and pulsed with 1 μCi of [^{3}H]leucine/well. After a 1 h incubation, protein synthesis was assayed as described in the Experimental section. The S.D. was calculated from the results obtained in three to six experiments.

Additions	Protein synthesis (%)
None	100
BS-RNase	63 ± 10
Monensin	85 ± 10
BS-RNase + monensin	35 ± 8
Nigericin	86 ± 10
BS-RNase + nigericin	33 ± 2

Since no receptors were identified in previous studies [9], and BS-RNase has been reported to permeate synthetic membranes, either uncharged or negatively charged [27], non-receptor-mediated endocytosis has been proposed [9].

The ECM/cell-membrane system would seem to constitute a selective station, however, as the immunofluoresence experiments described above on fibroblasts and thyroid cells have shown that RNase A, or MCM-BS-RNase, is not internalized [17]. The former is a monomeric non-cytotoxic RNase, whereas the latter is a monomeric, non-cytotoxic, but correctly folded and enzymically active form of BS-RNase. Anti-(RNase A) or anti-BS-RNase sera were used respectively to detect RNase A or monomeric BS-RNase. With these proteins no extra- or intracellular immunofluorescence signals were detectable in the treated cells (results not shown). These results indicate that, for monomeric non-cytotoxic RNases such as RNase A and MCM-BS-RNase respectively, very similar to, or identical in primary and three-dimensional structure with, a BS-RNase subunit, there is no extracellular binding nor internalization in normal or malignant cells. These data are in line with previous results [9] showing the absence of binding sites for MCM-BS-RNase or RNase A at the ECM of normal or malignant fibroblasts.

A dimeric variant of RNase A (PLCC-RNase AA), endowed with cytotoxic activity selective for malignant cells [5], was also tested in immunofluorescence experiments. In this dimeric RNase A variant, four residues of the wild-type protein are replaced with residues considered essential to the dimeric structure of BS-RNase, including the two cysteine residues involved in the intersubunit disulphide bonds. The results of immunofluorescence experiments, carried out on SVT2 cells as described above for BS-RNase, showed that, in contrast with its parent monomeric RNase A, the dimeric, cytotoxic variant of RNase A can effectively bind to the cell surface and is internalized in endosome-like particles, with immunofluorescence profiles identical with those shown in Figure 1 for BS-RNase (results not shown).

Taken together, these data on naturally dimeric BS-RNase and its non-cytotoxic monomeric counterpart, and on non-cytotoxic RNase A and its dimeric cytotoxic counterpart, are suggestive of the importance of a dimeric structure for the first steps in the mechanism of action of a cytotoxic RNase, i.e. for ECM binding and for internalization.

Intracellular routing of BS-RNase

The cytotoxic action of certain toxins, such as diphtheria toxin, measured by their effect on the protein synthesis, is strongly

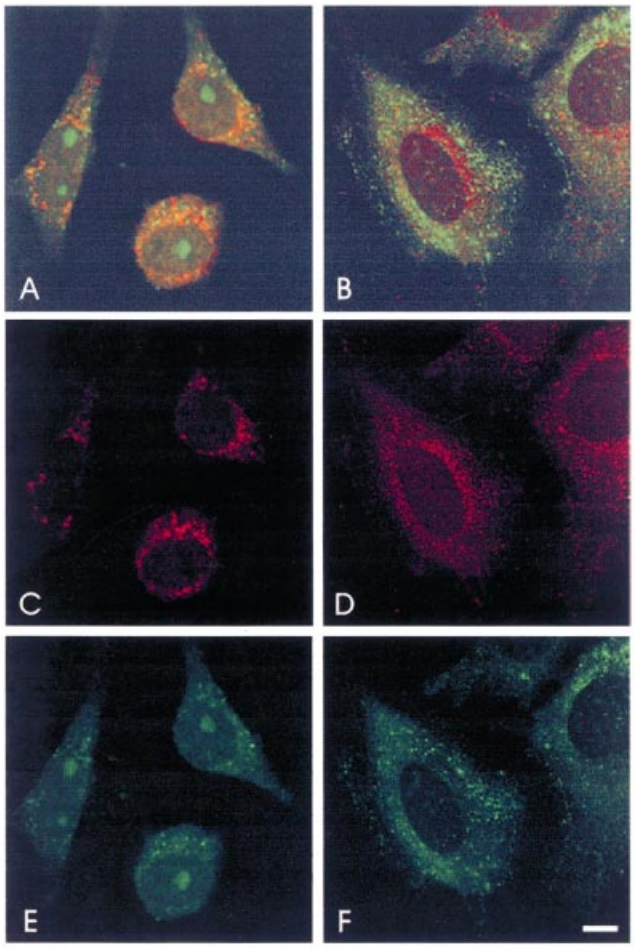

Figure 3 Fluorescence studies of co-localization of BS-RNase and a TGN marker

SVT2-transformed fibroblasts (**A**, **C** and **E**) and 3T3 normal fibroblasts (**B**, **D** and **F**) were treated with BS-RNase (50 μg/ml) for 3 h at 37 °C. After washings, fixation and permeabilization as described in the legend to Figure 1, cells were treated in sequence with: (1) antiserum anti-BS-RNase; (2) anti-rabbit fluorescein-conjugated secondary antibody; (3) a monoclonal anti-TGN38 antibody; (4) an anti-mouse rhodamine-conjugated secondary antibody. Images (**C**) and (**D**) show the immunofluorescence signal of TGN38; images (**E**) and (**F**) show the immunofluorescence signal of BS-RNase. The bar represents 10 μm.

inhibited by raising the endosomal pH with carboxylic ionophores such as monensin or nigericin [15,28]. These findings have led to conclude that a functional acidic endosomal compartment is essential for translocation of these toxins to the cytosol [15]. The possibility of a direct route for BS-RNase from endosomes to the cytosol was thus investigated by measuring the toxic effect of BS-RNase on protein synthesis in the presence or the absence of those ionophores. SVT2 cells were grown for 24 h, then treated for 16 h of growth with either ionophore alone (100 nM monensin or 10 nM nigericin) or with BS-RNase alone (50 μg/ml) or with BS-RNase in the presence of monensin or nigericin at their respective concentrations. Treated and untreated control cells were then incubated with [^{3}H]leucine (see the Experimental section). The results of those experiments (Table 1) indicate that monensin or nigericin do not inhibit the cytotoxic effect of BS-RNase; rather, they produce a modest increase of toxicity. It is likely that these ionophores, even at the low concentrations used in these experiments, may affect also the

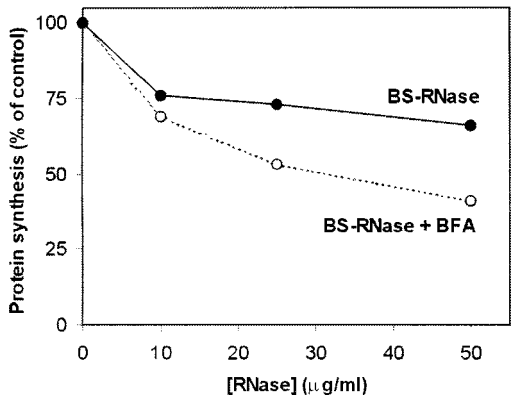

Figure 4 Cytotoxicity of BS-RNase tested with SVT2 cells in the absence or presence of BFA

SVT2 cells were plated at 5×10^4/well in 24-well plates in DMEM complete medium. After 24 h of growth, cells were treated with increasing concentrations of BS-RNase in the absence (●) or presence (○) of BFA (0.2 μg/ml) for 16 h at 37 °C. Protein synthesis was then assayed as described in the Experimental section.

Golgi structure [28]. The main conclusion from these experiments is that an inactivation of the endosomal function does not block the cytotoxic action of BS-RNase. Hence, the possibility of a direct translocation of internalized BS-RNase from endosomes to the cytosol may be excluded.

To investigate the further progressing of the protein in the cell, immunofluorescence and biochemical experiments were carried out. In the first approach, malignant SVT2 cells and 3T3 fibroblasts were treated for 3h at 37 °C with BS-RNase (50 μg/ml), and then with an antibody directed to the TGN [19]. Figures 3(A), 3(C) and 3(E) show a clear localization signal of the protein in the Golgi apparatus of malignant cells. In normal 3T3 cells instead (see Figures 3B, 3D and 3F), protein localization was found to be very similar to that obtained in the experiments illustrated in Figures 1 and 2, and no localization in the Golgi structure was detectable. Thus it is tempting to conclude that, in both normal and malignant cells, BS-RNase is endocytosed and internalized in endosomal vesicles, but only in malignant cells does it reach the Golgi apparatus.

It should be noted that, in these experiments, a nucleolar protein staining was also detectable, but only in malignant SVT2 cells. This is reminiscent of the previously reported nucleolar localization of angiogenin, a homologous RNase also endowed with a special bioaction, such as angiogenesis [29]. This finding is noteworthy, as it has been previously ascertained that only in malignant cells did the protein reach the cytosol [9]. Thus a nucleolar localization of BS-RNase in malignant SVT2 cells, and its absence in 3T3 cells, are indicative of a direct transport of the protein from the cytosolic to the nuclear compartment, which is consistent with its cytosolic localization, but only in malignant cells. The lack of a nuclear localization signal in the BS-RNase amino acid sequence may not exclude the transport of this protein to the nucleus. Similar cases have been reviewed previously [30].

For the biochemical approach, BFA was employed, a reagent reported to interfere with the endocytic pathway of cytotoxins, mainly by a disrupting action on the Golgi apparatus [15,31,32]. When BFA (0.1–0.2 μg/ml) was added to exponentially growing SVT2 cells treated for 16 h with 5–50 μg of BS-RNase/ml, an increase in the cytotoxic action of the RNase was consistently observed in a series of six experiments. A typical experiment is illustrated in Figure 4. It must be added that, at the highest concentration employed (0.2 μg/ml), BFA by itself was found to be only slightly cytotoxic to SVT2 cells, i.e. with an inhibitory effect on protein biosynthesis lower than 10%.

The conclusion that can be drawn from the enhancement induced by BFA of the cytopathic effect of BS-RNase on malignant fibroblasts is that the Golgi apparatus is involved as an intracellular station of the protein on its way to the cytosol. A similar conclusion was reached in previous studies [10] on the basis of the observation of an increased toxicity of the enzyme on rat glioma 9L cells in the presence of retinoic acid, a Golgi-disrupting agent. However, in the latter report, BFA was found to have no effect on the cytotoxic action of BS-RNase, even at concentrations 20-fold higher than that used in the present study. The different effects of BFA on the cytotoxic action of BS-RNase on malignant fibroblasts and glioma cells may be explained [31] by the different cell types under investigation, since they show different sensitivities to BFA alone. We found that, at concentrations higher than 1 μg/ml, BFA was dramatically toxic to SVT2 fibroblasts, whereas it did not affect glioma-cell viability even at 5 μg/ml.

Of greater interest is the finding that the cytopathic effect of BS-RNase is enhanced by BFA. This is a very uncommon BFA effect. BFA has been found [33] either to protect cells from cytotoxins such as ricin or *Pseudomonas* exotoxin, or to have no effect on cytotoxicity, as in the case of diphtheria toxin or Onconase™ [10,12]. The surprising finding that BFA increases BS-RNase cytotoxicity may have an explanation. It has been proposed [32] that BFA acts by restructuring the endocytic/exocytic vesicular cell system into two separate subsystems: one comprising the plasma membrane, endosomes and TGN, and the other comprising *medial*-Golgi, *cis*-Golgi and ER. Albeit still functioning, the two subsystems would not communicate with each other, and a discontinuity would occur between TGN and the remaining Golgi structures. It can thus be surmised that, in the presence of BFA, once BS-RNase reaches TGN, it may not proceed any further and hence it accumulates in the TGN, so that greater amounts of the protein reach the cytosol, where its toxic effects are exerted.

To verify this hypothesis and investigate the surprising enhancing effect of BFA on BS-RNase cytotoxicity, we prepared and tested a variant of BS-RNase engineered by inserting at its C-terminal end the -Lys-Asp-Glu-Leu (KDEL) sequence. The presence of the C-terminal KDEL signal in a protein sequence has been demonstrated to mark its localization to the ER [34]. Retrieval and transfer of KDEL-marked proteins from the Golgi apparatus to ER is carried out by the KDEL receptor, a carrier protein located in *cis*-Golgi, but also found as far as the TGN [35].

When the BS-RNase-KDEL variant was tested on malignant SVT2 fibroblasts at concentrations up to 10 μg/ml, it was found to have no cytotoxic effects on SVT2 cells. In fact a slight stimulating effect on protein synthesis was observed, whereas wild-type BS-RNase showed the expected toxicity (see Figure 5). However, when the concentration of BS-RNase-KDEL was raised above 10 μg/ml, its cytotoxic effect was apparent, although consistently lower than that of wild-type BS-RNase (see Figure 5). It should be noted that identical results (results not shown) were obtained when these experiments were carried out with BS-RNase-KDEL in the presence of BFA, under the same conditions as those described above for native BS-RNase.

These data led us to exclude the possibility that native BS-RNase travels as far as the ER and confirm that the Golgi apparatus, most likely the TGN, is an essential station in the intracellular pathway of BS-RNase. They also offer an ex-

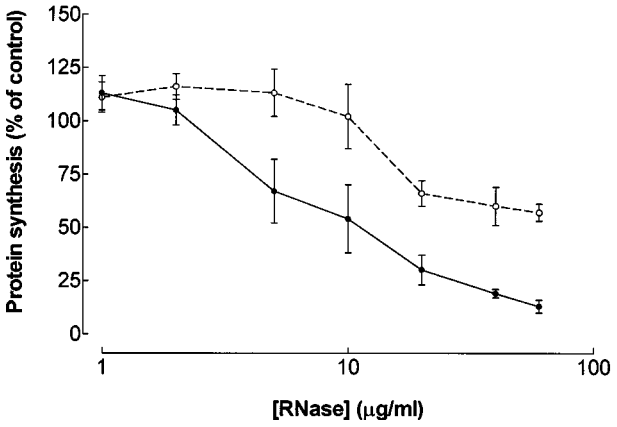

Figure 5 Cytotoxicity of BS-RNase and BS-RNase-KDEL to SVT2 cells

SVT2 cells (2.5×10^3/well) in 96-well plates were incubated with increasing concentrations of BS-RNase (●) or BS-RNase-KDEL (○). After a 48 h incubation, protein synthesis was assayed as described in the Experimental section. The S.D. was calculated from data obtained in three experiments.

planation for the biphasic dose–response curve of KDEL-BS-RNase. The lack of cytotoxicity of low concentrations of BS-RNase-KDEL, and its cytotoxic effect at higher concentrations, may be due to the limited availability of KDEL receptor, which can only handle limited amounts of RNase-KDEL for its transfer from the Golgi to the ER. Upon saturation of the receptor, the Golgi → ER route is shut off, so that the protein will increasingly accumulate and enter the cytosol to exert its cytotoxic effect. Cytotoxicity of KDEL-BS-RNase may be lower than that of wild-type BS-RNase, since a fraction of the ER-marked protein may be sequestered in the ER. The importance of TGN as a likely immediate pre-cytosol station of BS-RNase is confirmed by the increase in cytotoxicity also observed in the presence of BFA for the BS-RNase-KDEL variant.

Conclusions

The results described above illuminate some of the key stations encountered by BS-RNase in its journey from outside the cell to the cytosol. Immunofluorescence studies indicate that, in fibroblasts or in epithelial cells, the RNase concentrates on the cell surface, apparently at the ECM. It crosses the cell membrane and is packaged in endosomes. Although this route is followed by the cytotoxin in both normal and malignant cells, monomeric non-cytotoxic homologous RNases do not follow it, as they are not detected at the cell surface, or in endosomes. Consistently, a dimeric cytotoxic variant form of RNase A was found to follow the same pathway from the ECM through endosomes as naturally dimeric and cytotoxic BS-RNase.

The toxic effect of BS-RNase on protein synthesis is not blocked by ionophores that inactivate the endosomal function. This shows that a direct route from endosomes to cytosol is not likely.

From the endosomes the RNase reaches the TGN, and thence the cytosol, but only in malignant cells, as is shown by the results of immunofluorescence studies and experiments with BFA. This conclusion is in perfect agreement with the results obtained from previous experiments [9] in which BS-RNase was found to degrade rRNA and to reach the cytosol only in malignant cells. It is not clear how BS-RNase is transported from the TGN to the cytosol. Possibly its ability to permeate membranes [27] may lead the protein to cross the TGN membrane.

A nucleolar localization was also found only for malignant cells. However, as this station may be reached through the cytosol, it may be indicative of the fate of only a fraction of internalized enzyme. Other routes beyond the TGN, to the Golgi stack or further on towards the ER, may be open to the enzyme, but may not lead directly to the cytosol, hence they are not important for the enzyme's cytotoxic action. When these alternative routes are blocked, as in the presence of BFA, the enzyme toxicity increases. Consistently, when the protein is forced to travel through these alternative routes to the ER by marking it with an ER retrieval signal, its toxicity is abolished, as long as the retrieval KDEL receptor is available.

Taken together, these results concur to identify the cytotoxic pathway of seminal RNase as the following:

ECM → PM → endosomes → TGN → cytosol/nucleoli

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REFERENCES

1 D'Alessio, G., Di Donato, A., Mazzarella, L. and Piccoli, R. (1997) Seminal ribonuclease: the importance of diversity. In Ribonucleases: Structures and Functions (Riordan, J. F. and D'Alessio, G., eds.), pp. 383–423, Academic Press, New York
2 Matousek, J. (1973) The effect of bovine seminal ribonuclease (AS RNase) on cells of Crocker tumour in mice. Experientia **29**, 858–859
3 Youle, R. J. and D'Alessio, G. (1997) Antitumor RNases. In Ribonucleases: Structures and Functions (Riordan, J. F. and D'Alessio, G., eds.), pp. 491–509, Academic Press, New York
4 Vescia, S., Tramontano, D., Augusti Tocco, G. and D'Alessio, G. (1980) *In vitro* studies on selective inhibition of tumor cell growth by seminal ribonuclease. Cancer Res. **40**, 3740–3744
5 Di Donato, A., Cafaro, V. and D'Alessio, G. (1994) Ribonuclease A can be transformed into a dimeric ribonuclease with antitumor activity. J. Biol. Chem. **269**, 17394–17396
6 Leland, P. A., Schultz, L. W., Kim, B.-M. and Raines, R. T. (1998) Ribonuclease A variants with potent cytotoxic activity. Proc. Natl. Acad. Sci. U.S.A. **95**, 10407–10412
7 Suzuki, M., Saxena, S. K., Boix, E., Prill, R. J., Vasandani, V. M., Ladner, J. E., Sung, C. and Youle, R. J. (1999) Engineering receptor-mediated cytotoxicity into human ribonucleases by steric blockade of inhibitor interaction. Nat. Biotechnol. **17**, 265–270
8 Piccoli, R., Di Gaetano, S., De Lorenzo, C., Grauso, M., Monaco, C., Spalletti-Cernia, D., Laccetti, P., Cinatl, J., Matousek, J. and D'Alessio, G. (1999) A dimeric mutant of human pancreatic ribonuclease with selective cytotoxicity toward malignant cells. Proc. Natl. Acad. Sci. U.S.A. **96**, 7768–7773
9 Mastronicola, M. R., Piccoli, R. and D'Alessio, G. (1995) Key extracellular and intracellular steps in the antitumor action of seminal ribonuclease. Eur. J. Biochem. **230**, 242–249
10 Wu, Y., Saxena, S. K., Ardelt, W., Gadina, M., Mikulski, S. M., De Lorenzo, C., D'Alessio, G. and Youle, R. J. (1995) A study of the intracellular routing of the cytotoxic ribonucleases. J. Biol. Chem. **270**, 17476–17481
11 Mikulski, S. M., Chun, H. G., Mittelman, A., Panella, T., Puccio, C. A., Shogen, K. and Costanzi, J. J. (1995) Relationship between response rate and median survival in patients with advanced non-small cell lung cancer: comparison of onconase with other anticancer agents. Int. J. Oncol. **6**, 889–897
12 Newton, D. L., Boque, L., Wlodawer, A., Huang, C. Y. and Rybak, S. M. (1998) Single amino acid substitutions at the N-terminus of a recombinant cytotoxic ribonuclease markedly influence biochemical and biological properties. Biochemistry **37**, 5173–5183
13 Wu, Y., Mikulski, S. M., Ardelt, W., Rybak, S. M. and Youle, R. J. (1993) A cytotoxic ribonuclease. Study of the mechanism of onconase cytotoxicity. J. Biol. Chem. **268**, 10686–10693

14 Lin, J. J., Newton, D. L., Mikulski, S. M., Kung, H. F., Youle, R. J. and Rybak, S. M. (1994) Characterisation of the mechanism of cellular and cell free protein synthesis inhibition by an antitumor ribonuclease. Biochem. Biophys. Res. Commun. **204**, 156–162
15 Lord, M. J. and Roberts, L. M. (1998) Toxin entry: retrograde transport through the secretory pathway. J. Cell Biol. **140**, 733–736
16 Tamburrini, M., Piccoli, R., De Prisco, R., Di Donato, A. and D'Alessio, G. (1986) Fast and high-yelding procedures for the isolation of bovine seminal RNase. Ital. J. Biochem. **35**, 22–32
17 D'Alessio, G., Malorni, M. C. and Parente, A. (1975) Dissociation of bovine seminal ribonuclease into catalytically active monomers by selective reduction and alkylation of the intersubunit disulfide bridges. Biochemistry **14**, 1116–1122
18 Harlow, E. and Lane, D. (1988) Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19 Greenfield, J. P., Tsai, J., Gouras, G. K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S. S., Greengard, P. and Xu, H. (1999) Endoplasmic reticulum and *trans*-Golgi network generate distinct populations of Alzheimer β-amyloid peptides. Proc. Natl. Acad. Sci. U.S.A. **96**, 742–747
20 Ambesi-Impiombato, F. S., Parks, L. A. and Coon, H. G. (1980) Culture of hormone-dependent functional ephitelial cells from rat thyroids. Proc. Natl. Acad. Sci. U.S.A. **77**, 3455–3459
21 Portella, G., Ferulano, G., Santoro, M., Grieco, M., Fusco, A. and Vecchio, G. (1989) The Kirsten murine sarcoma virus induces rat thyroid carcinomas *in vivo*. Oncogene **4**, 181–188
22 Saelinger, C. B. (1988) Use of Exotoxin A to inhibit protein synthesis. Methods Enzymol. **165**, 226–231
23 Sambrook, K. J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
25 de Nigris, M., Russo, N., Piccoli, R., D'Alessio, G. and Di Donato, A. (1993) Expression of bovine seminal ribonuclease in Escherichia coli. Biochem. Biophys. Res. Commun. **193**, 155–160
26 Kunitz, M. (1946) A spectrophotometric method for the measurement of ribonuclease activity. J. Biol. Chem. **164**, 563–568
27 Mancheño, J. M., Gasset, M., Onaderra, M., Gavilanes, J. G. and D'Alessio, G. (1994) Bovine seminal ribonuclease destabilizes negatively charged membranes. Biochem. Biophys. Res. Commun. **199**, 119–124
28 Mellman, I., Fuchs, R. and Helenius, A. (1986) Acidification of endocytic and exocytic pathways. Annu. Rev. Biochem. **55**, 663–700
29 Moroianu, J. and Riordan, J. F. (1994) Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity. Proc. Natl. Acad. Sci. U.S.A. **91**, 1677–1681
30 Nigg, E. A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature (London) **386**, 779–787
31 Pelham, H. R. B. (1991) Multiple targets for brefeldin A. Cell **67**, 449–451
32 Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. and Klausner, R. D. (1991) Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell **67**, 601–616
33 Yoshida, T., Chen, C. C., Zhang, M. S. and Wu, H. C. (1991) Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and *Pseudomonas* toxin. Exp. Cell Res. **192**, 389–395
34 Pelham, H. R. B. (1990) The retention signal for soluble proteins of the endoplasmic reticulum. Trends Biochem. Sci. **15**, 483–486
35 Miesenbock, G. and Rothman, J. E. (1995) The capacity to retrieve escaped ER proteins extends to the *trans*-most cisterna of the Golgi stack. J. Cell Biol. **129**, 309–319

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